

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C07K 14/575, A61K 38/22	A1	(11) International Publication Number: WO 96/29342 (43) International Publication Date: 26 September 1996 (26.09.96)
(21) International Application Number: PCT/DK96/00106 (22) International Filing Date: 18 March 1996 (18.03.96) (30) Priority Data: 0275/95 17 March 1995 (17.03.95) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): JONASSEN, Ib [DK/DK]; Kirkevænget 2, DK-2500 Valby (DK). HAVELUND, Svend [DK/DK]; Kurvej 38, DK-2880 Bagsværd (DK). HANSEN, Peter, Hertz [DK/DK]; Nybrovej 222, DK-2800 Lyngby (DK). KURTZHALS, Peter [DK/US]; Apartment A411, 20 Chapel Street, Brookline, MA 02146 (US). HALSTRØM, John, Broberg [DK/DK]; Søndergade 44, DK-3390 Hundested (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).	(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	

(54) Title: LIPOPHILIC PEPTIDE HORMONE DERIVATIVES**(57) Abstract**

A pharmacologically active peptide hormone derivative in which the parent peptide hormone has been modified by introducing either a lipophilic substituent, W, in the N-terminal amino acid or a lipophilic substituent, Z, in the C-terminal amino acid of the parent peptide hormone or an analogue thereof, said lipophilic substituent having from 8 to 40 carbon atoms, has a protracted profile of action.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

Lipophilic peptide hormone derivatives.

FIELD OF THE INVENTION

The present invention relates to novel derivatives of peptide hormones and analogues thereof which have a protracted profile of action and to methods of making and using them.

BACKGROUND OF THE INVENTION

Peptide hormones are widely used in medical practice and since they can be produced by recombinant DNA technology it can be expected that their importance will increase also in the years to come. When native peptide hormones or analogues thereof are used in therapy it is generally found that they have a high clearance rate. A high clearance rate of a therapeutic agent is inconvenient in cases where it is desired to maintain a high blood level thereof over a prolonged period of time since repeated administrations will then be necessary. Examples of peptide hormones which have a high clearance rate are: ACTH, corticotropin-releasing factor, angiotensin, calcitonin, insulin and fragments and analogues thereof, glucagon, glucagon-like peptide and analogues and fragments thereof, IGF-1, IGF-2, enterogastrin, somatostatin, somatotropin, somatomedin, parathyroid hormone, thrombopoietin, erythropoietin, hypothalamic releasing factors, prolactin, thyroid stimulating hormones, endorphins, enkephalins, vasopressin, oxytocin, opioids and analogues thereof, superoxide dismutase, interferon, asparaginase, arginase, arginine deaminase, adenosine deaminase and ribonuclease.

Although it has in some cases been possible to influence the release profile of peptide hormones by applying suitable pharmaceutical compositions this approach has various shortcomings and is not generally applicable. Accordingly, there still is a need for improvements in the field of administration of peptide hormones.

SUMMARY OF THE INVENTION

In the present text, the term peptide is used to designate both small peptides and polypeptides and proteins. The terms peptide and peptide hormone are used as encompassing both naturally
5 occurring and synthetic peptide hormones and fragments and analogues thereof. Analogues are peptides in which one or more amino acids in the parent peptide have been deleted or substituted by another amino acid, or to which one or more amino acids have been added, and which still have qualitatively
10 - but not necessarily quantitatively - the same pharmacological effect as the parent peptide.

The present invention relates generally to novel derivatives of peptide hormones which have a protracted profile of action.

Thus, in its broadest aspect, the present invention relates to
15 a pharmacologically active peptide hormone which has been modified by introducing a lipophilic substituent comprising from 8 to 40 carbon atoms in either the N-terminal or the C-terminal amino acid of the native peptide hormone or an analogue thereof, with the proviso that when the lipophilic
20 substituent is attached to the N-terminal amino group then the substituent comprises a group which can be negatively charged and with the further proviso, that said peptide hormone is not insulin or an analogue thereof.

In one preferred embodiment of the present invention, a
25 carboxyl group contained in the lipophilic group, W, forms an amide bond together with the α -amino group of the N-terminal amino acid of the parent peptide.

In another preferred embodiment of the present invention, a
30 amide bond together with the ϵ -amino group of a N-terminal lysine.

In another preferred embodiment of the present invention, the lipophilic group, W, is composed of a spacer and a bulk lipophilic substituent. The spacer is preferably succinic acid, Glu or Asp. The bulk lipophilic substituent is preferably a
5 straight chain fatty acid which optionally has an amino group. When succinic acid is used as spacer, one of its carboxyl groups forms an amide bond with an amino group in the N-terminal amino acid of the parent peptide while the other carboxyl group forms an amide bond with an amino group
10 contained in the bulk lipophilic group. When Glu or Asp is used as spacer, one of the carboxyl groups forms an amide bond with an amino group in the N-terminal amino acid of the parent peptide while the bulk lipophilic substituent preferably is the acyl group of a straight chain fatty acid or of an acid which
15 comprises a partly or completely hydrogenated cyclopentanophenanthrene skeleton which acyl group is attached to the amino group of the spacer.

In another preferred embodiment of the present invention, an amino group contained in the lipophilic group Z forms an amide
20 bond together with the carboxyl group of the C-terminal amino acid of the parent peptide.

In another preferred embodiment of the present invention, Z is a straight chain fatty acid which has an amino group.

In another preferred embodiment of the present invention, Z has
25 a group which can be negatively charged.

In another preferred embodiment of the present invention, Z has a free carboxylic acid group.

In another preferred embodiment of the present invention, the lipophilic group Z is composed of a spacer and a bulk
30 lipophilic substituent. The spacer is preferably Lys, Glu or Asp. When Lys is used as spacer, the bulk lipophilic substituent, in one preferred embodiment, is the acyl group of a straight chain fatty acid or of an acid which comprises a

partly or completely hydrogenated cyclopentanophenanthrene skeleton which acyl group is attached to the amino group of the spacer group. In a further preferred embodiment, when Lys is used as spacer, a further spacer is inserted between the ϵ -5 amino group of Lys and the bulk lipophilic substituent. In one preferred embodiment, such a further spacer is succinic acid which forms an amide bond with the ϵ -amino group of Lys and with an amino group present in the bulk lipophilic substituent. In another preferred embodiment such a further spacer is Glu or 10 Asp which form one amide bond with the ϵ -amino group of Lys and a further amide bond with a carboxyl group present in the bulk lipophilic substituent which is preferably a straight chain fatty acid or an acid which comprises a partly or completely hydrogenated cyclopentanophenanthrene skeleton.

15 In another preferred embodiment, the present invention relates to the use of the peptide derivatives of the invention as medicaments.

In another preferred embodiment, the present invention relates to medicaments containing the peptide derivatives of the 20 invention.

In another preferred embodiment, the present invention relates to the a pharmaceutical composition for the treatment of osteoporosis in a patient in need of such a treatment, comprising a therapeutically effective amount of an IGF-1 25 derivative according to the invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the present invention relates to a method of treating osteoporosis in a patient in need of such a treatment comprising administering to the patient a 30 therapeutically effective amount of an IGF-1 derivative according to the invention together with a pharmaceutically acceptable carrier.

Examples of parent peptide hormones which are of interest in

connection with the present invention are the following: ACTH, corticotropin-releasing factor, angiotensin, calcitonin, glucagon, glucagon-like peptide and analogues and fragments thereof e.g. GLP-1 and GLP-2 and analogues and fragments thereof, IGF-1, IGF-2, enterogastrin, somatostatin, somatotropin, somatomedin, parathyroid hormone, thrombopoietin, erythropoietin, hypothalamic releasing factors, prolactin, thyroid stimulating hormones, endorphins, enkephalins, vasopressin, oxytocin, opioids and analogues thereof, superoxide dismutase, interferon, asparaginase, arginase, arginine deaminase, adenosine deaminase and ribonuclease.

Examples of particularly preferred derivatives of IGF-1 and IGF-1 analogues are:

- Lys⁶⁸(N'-tetradecanoyl) des(69,70) human IGF-1;
- 15 Lys⁶⁸[N'-γ-Glu(N"-hexadecanoyl)-OH]-OH des(69,70) human IGF-1;
- Lys⁶⁹(N'-tetradecanoyl) des(70) human IGF-1;
- Ser⁶⁹-NH(CH₂)_nCOOH des(70) human IGF-1 wherein n is an integer from 12 to 24;
- Ser⁶⁹-NH(CH₂)_nCH₃ des(70) human IGF-1 wherein n is an integer
- 20 from 12 to 24;
- Lys⁷¹(N'-tetradecanoyl) human IGF-1;
- Ala⁷⁰-NH(CH₂)_nCOOH human IGF-1 wherein n is an integer from 12 to 24; and
- Ala⁷⁰-NH(CH₂)_nCH₃ human IGF-1 wherein n is an integer from 12 to
- 25 24.

A preferred derivative of somatostatin is:

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Thr-Ser-Cys-Lys[N'-γ-Glu(N"-tetradecanoyl)-OH]-OH (the two Cys residues are connected via a disulphide bridge).

30 A preferred derivative of GLP-1 is:

His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys[N'-γ-Glu(N"-tetradecanoyl)-OH]-OH.

A preferred ANP analogue is:

Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-Lys[N'- γ -Glu(N^o-tetradecanoyl)-OH]-OH.

5 A preferred type of derivative of a dynorphin analogue is:

Tyr-Gly-Gly-Phe-Cys-Arg-Arg-D-Ala-Arg-Pro-Cys-NH-(CH₂)_n-COOH, wherein n is an integer from 8 to 24.

A preferred derivative of enterogastrin is:

H-Ala-Pro-Gly-Pro-Arg-Lys(N'-tetradecanoyl)-OH.

10 DETAILED DESCRIPTION OF THE INVENTION

Pharmaceutical compositions

Pharmaceutical compositions containing a peptide derivative according to the present invention may be administered parenterally to patients in need of such a treatment.
15 Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a
20 liquid for the administration of the peptide derivative in the form of a nasal spray. As a still further option, it may also be possible to administer the peptide derivatives transdermally.

Pharmaceutical compositions containing a compound of the
25 present invention may be prepared by conventional techniques, e.g. as described in Remington's Pharmaceutical Sciences, 1985.

Thus, the injectable compositions of the peptide derivatives of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and
30 mixing the ingredients as appropriate to give the desired end

product.

Thus, according to one procedure, the peptide derivative is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted - if necessary - using an acid, e.g. hydrochloric acid, or a base, e.g. aqueous sodium hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

Examples of isotonic agents are sodium chloride, mannitol and glycerol.

Examples of preservatives are phenol, m-cresol, methyl p-hydroxybenzoate and benzyl alcohol.

Examples of suitable buffers are sodium acetate and sodium phosphate.

A composition for nasal administration of certain peptide hormones may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S).

The peptide derivatives of this invention can be used in the treatment of various diseases. The particular peptide derivative to be used and the optimal dose level for any patient will depend on the disease to be treated and on a variety of factors including the efficacy of the specific peptide derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case. It is recommended that the dosage of the peptide derivative of this invention be determined for each individual patient by those skilled in the art in a similar way as for known peptide hormones.

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both 5 separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

Abbreviations:

Fmoc	:	9-fluorenylmethyloxycarbonyl.
10 For	:	formyl
Dde	:	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-ethyl.
DMF	:	N,N-dimethylformamide.
Tbu	:	tert-butyl.
Acm	:	acetamidomethyl.
15 DIC	:	N,N'-diisopropylcarbodiimide.
HOBT	:	1-hydroxybenzotriazole.
TFA	:	trifluoroacetic acid.

Analytical

Molecular masses of the products prepared were obtained by 20 plasma desorption mass spectrometry (PDMS) using Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden).

Determination of lipophilicity.

The lipophilicity of peptides and peptide derivatives relative to human insulin, k'_{rel} , was measured on a LiChrosorb RP18 (5 μ m, 25 4x250 mm) HPLC column by isocratic elution at 40°C using mixtures of A) 0.1 M sodium phosphate buffer, pH 7.3, containing 10% acetonitrile, and B) 50% acetonitrile in water as eluents. The elution was monitored by following the UV

absorption of the eluate at 214 nm. Void time, t_0 , was found by injecting 0.1 mM sodium nitrate. Retention time for human insulin, t_{insulin} , was adjusted to at least $2t_0$ by varying the ratio between the A and B solutions. $k'_{\text{rel}} = (t_{\text{derivative}} - t_0) / (t_{\text{insulin}} - 5 t_0)$.

EXAMPLE 1

Synthesis of For-Nle-Leu-Phe-Nle-Tyr-Lys(N'-tetradecanoyl)-OH.

For-Nle-Leu-Phe-Nle-Tyr-Lys-OH, was purchased from Bachem
10 Feinchemikalien AG, Switzerland. The peptide is a potent chemo-attractant for human neutrophils. The title compound was prepared by dissolving 17 mg of For-Nle-Leu-Phe-Nle-Tyr-Lys-OH in 5 ml of DMF and then adding 35 μ l of triethylamine followed by 20 mg of solid tetradecanoic acid succinimidyl-N-hydroxy ester
15 to the solution. The reaction was monitored by RP-HPLC employing a column packed with reversed phase C18 silica material. For the elution was used a gradient from 30% ethanol to 80 % ethanol in 0.1% aqueous TFA. The product was purified on a column (length 250 mm diameter 20 mm) packed with C18
20 silica reversed phase material. The compound was dissolved in 74% ethanol/0.1% aqueous TFA and subsequently applied to the column and purified at 40 °C by isocratic elution in the same buffer at a flow rate of 6 ml/hour. The yield was 20 mg. The identity of the compound was confirmed by PDMS.

25 Molecular mass, found by PDMS: 1034, theory: 1034.

The lipophilicity of the title compound relative to human insulin was found to be 8.2×10^3 .

Reference

The reference compound, For-Nle-Leu-Phe-Nle-Tyr-Lys-OH, was
30 purchased from Bachem Feinchemikalien AG, Switzerland, and used as received. The lipophilicity of the reference compound relative to human insulin was found to be 2.3.

EXAMPLE 2

Synthesis of H-Tyr-D-Ala-Gly-Phe-Leu-Lys(N'tetradecanoyl)-OH.

The enkephalin derivative H-Tyr-D-Ala-Gly-Phe-Leu-Lys(N'-5 tetradecanoyl)-OH was made from Boc-Tyr-D-Ala-Gly-Phe-Leu-Lys-OH (A-2435 Bachem Feinchemikalien AG, Switzerland). The Boc-Tyr-D-Ala-Gly-Phe-Leu-Lys-OH was acylated using tetradecanoic acid succinimidyl-N-hydroxy ester as described in Example 1. The reaction mixture was evaporated to dryness and the residue 10 was dissolved in TFA and evaporated to dryness, solubilized in ethanol/water/0.1% and purified by RP-HPLC as described in Example 1. The yield was 15 mg.

Molecular mass, found by PDMS: 909, theory: 907.

The lipophilicity of the title compound relative to human 15 insulin was found to be 2.3×10^3 .

Reference

The reference compound, H-Tyr-D-Ala-Gly-Phe-Leu-Lys-OH, was synthesized from Boc-Tyr-D-Ala-Gly-Phe-Leu-Lys-OH by dissolving 20 mg of this compound in 200 μ l of TFA and evaporating 20 to dryness. The residue was dissolved in 5% acetic acid and freeze dried. The lipophilicity of the reference compound relative to human insulin was found to be 3.0×10^{-3} .

EXAMPLE 3

Synthesis of H-Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys(N'-25 tetradecanoyl)-OH.

Fmoc-Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys-OH (obtained from Bachem Feinchemikalien AG, Switzerland) which is a potent inhibitor of renin was allowed to react with tetradecanoic acid 30 succinimidyl-N-hydroxy ester as described in Example 1. After

the acylation reaction, the Fmoc group was removed by addition of piperidine to the reaction mixture to a final concentration of 20%. The title compound was isolated by RP-HPLC as described in Example 1. The yield was 23 mg.

5 Molecular mass, found by PDMS: 1529.6, theory: 1529.

The lipophilicity relative to human insulin was found to be 5.3×10^3 .

Reference

The reference compound, H-Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-
10 Lys-OH, was synthesized from Fmoc-Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys-OH (obtained from Bachem Feinchemikalien AG, Switzerland). Thus, 20 mg of Fmoc-Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys-OH was dissolved in 500 μ l of 20% piperidine in DMF and left for 20 min. The reference compound was purified by RP-
15 HPLC as described in Example 1.

The lipophilicity of the reference compound relative to human insulin was found to be 2.3×10^2 .

EXAMPLE 4

Synthesis of Arg⁴,Arg⁹,Lys¹⁵(N'-tetradecanoyl) somatostatin.

20

The title compound was synthesized from Fmoc-Arg⁴,Arg⁹,Lys¹⁵ somatostatin which was obtained from Saxon Biochemicals GMBH, Hannover, Germany. 50 mg of Fmoc-Arg⁴,Arg⁹,Lys¹⁵ somatostatin was dissolved in a mixture of 346 μ l of DMF and 53.9 μ l of 4-
25 methymorpholine. The mixture was cooled to 15 °C and 15.9 mg of tetradecanoic acid succinimidyl-N-hydroxy ester dissolved in 100 μ l of DMF was added. The reaction was allowed to proceed for 3 hours and 20 min and then stopped by addition of 4140 μ l of 5% acetic acid in DMF. The title compound was purified by
30 RP-HPLC as follows: The sample was applied to a column (10x250 mm) of Lichrosorb RP-18 (7 μ m) Merck, Germany, Art. 9394. The

column was equilibrated with a mixture of 90% buffer A (50 mM tris, 75 mM $(\text{NH}_4)_2\text{SO}_4$, adjusted to pH 7.0 with H_2SO_4 , 20% CH_3CN) and 10% of buffer B (80% CH_3CN). The sample was applied to the column and eluted with a linear gradient from 10% to 90 % of 5 buffer B in buffer A at a flow rate of 4 ml/hour at 40 °C. The fractions containing the title compound were evaporated to dryness, dissolved in 50% acetic acid and desalted by gel filtration at 4 °C employing of column (16x150 mm) of BIO GEL P2 (BIO RAD, California, USA). The fractions containing the 10 desired product were diluted with water and freeze dried. The yield was 2 mg. The identity of the compound was confirmed by PDMS.

Molecular mass, found by PDMS: 2033, theory 2032.

Determination of protraction in pigs

15 The title peptide derivative of Example 4 was ^{125}I -labelled with Boulton & Hunters reagent (Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133. 529-539) as follows : 50 nmol of peptide was dissolved in 1 ml of DMSO and subsequently 400 μl of DMF and 2 μl of N-ethylisopropylamine were added. The solution was added 20 to an amount of Boulton & Hunters reagent containing 500 μCi of radioactivity. The reaction was allowed to proceed for 20 min. and then 10 μl of ethanolamine in DMF was added. The polypeptide was purified and isolated by RP-HPLC employing a column (4x250 mm) at a flow rate of 1 ml/min as described 25 above.

As a measure of the protraction, the disappearance rate in pigs was studied and $T_{50\%}$ was determined. $T_{50\%}$ is the time when 50% of the ^{125}I -labelled peptide has disappeared from the site of injection as measured with an external γ -counter (Ribet, U et 30 al., The Pig as a Model for Subcutaneous Absorption in Man. In: M. Serrano-Rios and P.J. Lefebvre (Eds): Diabetes 1985; Proceedings of the 12th Congress of the International Diabetes Federation, Madrid, Spain, 1985 (Excerpta Medica, Amsterdam, (1986) 891-96).

Subcutaneous injection of the ^{125}I -labelled peptide derivative in pigs showed a $T_{50\%}$ of 1.7 ± 0.5 h ($n=4$), whereas the non tetradecanoylated, ^{125}I -labelled reference peptide showed a $T_{50\%}$ of 0.7 ± 0.1 h.

5 Reference

The ^{125}I -labelled reference peptide was synthesized from Fmoc-Arg⁴, Arg⁹, Lys¹⁵ somatostatin. Thus, 20 mg of Fmoc-Arg⁴, Arg⁹, Lys¹⁵ somatostatin was dissolved in 1000 μl of 20% piperidine/DMF. After 20 min the product was purified by RP-HPLC, desalted and
10 freeze dried and labelled with Boulton & Hunters reagent as described in Example 4.

EXAMPLE 5

Synthesis of Lys¹⁵ (N'-tetradecanoyl) atrial natriuretic peptide.

15 Human (H-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-Lys(N'-tetradecanoyl)-COOH) was synthesized by standard Fmoc solid phase peptide synthesis (Methods in Molecular Biology, Vol 35: Peptide Synthesis Protocols). The ϵ -amino group of the
20 C-terminal lysine was acylated using tetradecanoic acid succinimidyl-N-hydroxy ester according to the procedure described below. The synthesis was performed manually in polypropylene syringes, on a resin based on a low cross linked polystyrene backbone grafted with polyoxyethylene (TentaGel Resin).

25 Procedure:

One gram of resin was added 3 equivalents of the acid labile linker 4-hydroxymethylphenoxyacetic acid (HMPA). 3 equivalents of Fmoc-Lys(Dde)-OH was coupled as the first amino acid, with 0.5 equivalent of 4-dimethylaminopyridine as activating
30 reagent. The Fmoc-protecting group was cleaved with 20% piperidine/DMF for 30 minutes. All other amino acids were coupled as N^o-Fmoc protected amino acids with a mixture of

DIC/HOBT (1:1 eq) in DMF as activating reagents. The amino acid Cys, was coupled as Fmoc-Cys(Acm)-OH. The cysteines were deprotected and oxidized by treatment with 10 Mm Iodine in DMF for 2 minutes. After the last Fmoc-protecting group was removed, the N^ε-group of the last coupled amino acid was protected with the Boc group by coupling with 5 equivalents of di-tert-butyl-dicarbonate. The Dde-protecting group of N^ε-Lys was cleaved with 2% hydrazine/DMF for 20 minutes, and the free N^ε-group was acylated with 5 equivalents of tetradecanoic acid succinimidyl-10 N-hydroxy ester. The Boc-, tBu-protecting groups and the HMPA-linker were cleaved with 95% TFA/5% H₂O for 1.5 hour. The TFA/H₂O was evaporated under reduced pressure, and the peptide was precipitated in diethyl ether as the HCl-salt, and freeze dried from a 10 mM ammonium hydrogen carbonate (pH 8.8). The 15 overall yield was 35 mg. By N-terminal sequencing the product was shown to have the correct sequence.

Molecular mass, found by PDMS: 3417, which corresponds to the calculated mass plus sodium.

EXAMPLE 6

20 Lys³⁰(N^ε-decanoyl) glucagon.

The title compound was purchased from Saxon Biochemicals GMBH, Hannover, Germany, as custom synthesis.

4.32 mg Lys³⁰(N^ε-decanoyl), glucagon (equivalent to 4 mg 25 glucagon) was dissolved in 4 ml of 1.8 mM hydrochloric acid added 0.9% sodium chloride and pH of the solution was measured to 2.7. The resulting solution was sterilized by filtration and transferred to a vial.

Two groups of rabbits (n=6 in each) were injected with 2 30 IU/animal of Insulin Actrapid at time -60 min. At time t=0 group 1 was injected SC with molar equivalent of 0.54 mg of Lys³⁰(N^ε-decanoyl) glucagon/rabbit and group 2 injected SC with

0.5 mg of glucagon/rabbit. Blood was sampled at times: -60, 0, 15, 30, 60, 120, 180 and 240 min, and the glucose concentration determined by the hexokinase method. The resulting blood glucose concentrations are given in the table in mg glucose/100 5 ml:

min	-60	0	15	30	60	120	180	240
glucagon	98	49	93	102	111	94	88	67
glucagon derivative	94	51	79	93	114	112	116	110

10 As it appears from the table, the blood glucose raising effect of glucagon is retained in Lys³⁰(Nε-decanoyl) glucagon but with a prolonged action.

CLAIMS

1. A pharmacologically active peptide hormone derivative in which the parent peptide hormone has been modified by introducing either a lipophilic substituent, W, in the N-
5 terminal amino acid or a lipophilic substituent, Z, in the C-terminal amino acid of the parent peptide hormone or an analogue thereof, said lipophilic substituent having from 8 to 40 carbon atoms, with the proviso that when the lipophilic substituent is attached to the N-terminal amino group then the
10 substituent comprises a group which can be negatively charged and with the further proviso, that said peptide hormone is not insulin or an analogue thereof.
2. A peptide hormone derivative according to claim 1 wherein a lipophilic group, W, is present.
- 15 3. A peptide hormone derivative according to claim 2 wherein W has from 12 to 35 carbon atoms.
4. A peptide hormone derivative according to claim 1 wherein a lipophilic group, Z, is present.
5. A peptide hormone derivative according to claim 4 wherein Z
20 has from 12 to 35 carbon atoms.
6. A peptide hormone derivative according to claim 1 wherein the parent peptide hormone is selected from the group consisting of ACTH, corticotropin-releasing factor, angiotensin, calcitonin, glucagon, glucagon-like peptide and
25 analogues and fragments thereof, IGF-1, IGF-2, enterogastrin, somatostatin, somatotropin, somatomedin, parathyroid hormone, thrombopoietin, erythropoietin, hypothalamic releasing factors, prolactin, thyroid stimulating hormones, endorphins, enkephalins, vasopressin, oxytocin, opioids and analogues
30 thereof, superoxide dismutase, interferon, asparaginase, arginase, arginine deaminase, adenosine deaminase and ribonuclease.

7. A peptide hormone derivative according to claim 2 wherein a carboxyl group contained in W forms an amide bond together with the α -amino group of the N-terminal amino acid.

8. A peptide hormone derivative according to claim 2 wherein a carboxyl group contained in W forms an amide bond together with the ϵ -amino group of a N-terminal lysine.

9. A peptide hormone derivative according to claim 2 wherein W is $\text{CH}_3(\text{CH}_2)_n((\text{CH}_2)_m\text{COOH})\text{CHNH-CO}(\text{CH}_2)_r\text{CO-}$ where n and m are integers and W has from 8 to 40, preferably from 12 to 35 carbon atoms.

10. A peptide hormone derivative according to claim 2 wherein W is a group of the general formula $\text{CH}_3(\text{CH}_2)_r\text{CO-NHCH}(\text{COOH})(\text{CH}_2)_s\text{CO-}$ wherein r is an integer from 10 to 24.

11. A peptide hormone derivative according to claim 2 wherein W is a group of the general formula $\text{CH}_3(\text{CH}_2)_r\text{CO-NHCH}((\text{CH}_2)_s\text{COOH})\text{CO-}$ wherein s is an integer from 8 to 24.

12. A peptide hormone derivative according to claim 4 wherein an amino group contained in Z forms an amide bond together with carboxyl group of the C-terminal amino acid.

13. A peptide hormone derivative according to claim 4 wherein Z is a group of the general formula $-\text{NHCH}(\text{COOH})(\text{CH}_2)_4\text{NH-CO}(\text{CH}_2)_m\text{CH}_3$ wherein m is an integer from 8 to 18, that is, Z is a N'-acylated lysine residue.

14. A peptide hormone derivative according to claim 4 wherein Z is a group of the general formula $-\text{NHCH}(\text{COOH})(\text{CH}_2)_4\text{NH-COCH}((\text{CH}_2)_p\text{COOH})\text{NH-CO}(\text{CH}_2)_q\text{CH}_3$ wherein p is an integer from 10 to 16.

15. A peptide hormone derivative according to claim 4 wherein Z is a group of the general formula $-\text{NHCH}(\text{COOH})(\text{CH}_2)_4\text{NH-}$

$\text{CO}(\text{CH}_2)_q\text{CH}(\text{COOH})\text{NH}-\text{CO}(\text{CH}_2)_q\text{CH}_3$, wherein q is an integer from 10 to 16.

16. A peptide hormone derivative according to claim 4 wherein Z is a group of the general formula $-\text{NHCH}(\text{COOH})(\text{CH}_2)_t\text{NH}-\text{CO}(\text{CH}_2)_t\text{CH}(\text{COOH})\text{NHCO}(\text{CH}_2)_t\text{CH}_3$, wherein t is zero or an integer from 1 to 22.

17. A peptide hormone derivative according to claim 4 wherein a spacer in the form of the dipeptide Gly-Lys has been inserted between the lipophilic group Z and the parent peptide hormone.

10 18. A peptide hormone derivative according to claim 4 wherein Z comprises a partly or completely hydrogenated cyclopentanophenanthrene skeleton.

19. A method of providing a pharmacologically active peptide hormone derivative which has a protracted profile of action
15 relative to the parent peptide hormone which method comprises modifying the parent peptide hormone by introducing either a lipophilic substituent, W , in the N-terminal amino acid or a lipophilic substituent, Z , in the C-terminal amino acid of the parent peptide hormone, said lipophilic substituent having from
20 8 to 40 carbon atoms, with the proviso that when the lipophilic substituent is attached to the N-terminal amino group then the substituent comprises a group which can be negatively charged and with the further proviso, that said peptide hormone is not insulin or an analogue thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00106

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/575, A61K 38/22
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, WPIL, CA, SCI SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 73, Embase, Dialog Accession No: 8421240, Embase No: 92097176, Muranishi S. et al: "Trials of lipid modification of peptide hormones for intestinal delivery"; & J. Control. Release (Netherlands), 1992, 19/1-3 (179-188)	1-19
X	Dialog Information Services, File 434, Sci Search, Dialog accession No: 12616071, Tenma T et al: "Development of new lipophilic derivatives of tetra- gastrin - physicochemical characteristics and intes- tinal-absorption of acyl-tetragastrin derivatives in rats"; & Pharmaceutical Research, 1993, V10, N10 (OCT), P1488-1492	1-19

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *B* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z document member of the same patent family

Date of the actual completion of the international search

28 June 1996

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Date of mailing of the international search report

02-07-1996

Authorized officer

Carolina Palmcrantz
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00106

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 434, Sci Search, Dialog Accession No: 13565514, Yodoya E et al: "Enhanced Permeability of Tetra- gastrin Across the Rat Intestinal-Membrane and its reduced degradation by Acylation with Various Fatty-Acids"; & Journal of Pharmacology and Experimental Therapeutics, 1994, V271, N3 (DEC), P1509-1513 --	1-19
X	Chemical Abstracts, Volume 123, No 10, 4 Sept 1995 (04.09.95), (Columbus, Ohio, USA), Cruz, M.E.M. et al, "Native and lipophilic derivatives of asparaginase and superoxide dismutase and respective liposomal forms", page 749, THE ABSTRACT No 122862m, Proc.Int.Symp.Contr.Release Bioact.Mater1994, 21ST, 346-347 --	1-19
X	Chemical Abstracts, Volume 118, No 16, 19 April 1993 (19.04.93), (Columbus, Ohio, USA), Martins, M.B. et al, "Lipophilic derivatives of copper-zinc-superoxide dismutase: Characterization and immobilization in liposomes", page 440, THE ABSTRACT No 154346j, Proc.Int.Symp.Contr.Release Bioact.Mater1992, 19th, 524-525 -----	1-19